Association of Genetic Variants in the Calcium-Sensing Receptor with Risk of Colorectal Adenoma

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Abstract

Objective: **Evidence suggests that calcium prevents colorectal cancer, possibly mediated through the calcium-sensing receptor (CASR). We assessed the associations between *CASR* gene variants and risk for colorectal adenoma, a cancer precursor. We further investigated gene-diet interactions between the *CASR* variants and calcium intake on adenoma risk.**

Methods: **Individuals with advanced distal adenomas (n = 716) and controls with a negative sigmoidoscopy exam (n = 729) were randomly selected from participants in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Three nonsynonymous variants in the intracellular signaling region of *CASR* (A986S, R990G, Q1011E) were analyzed by Taqman.**

Results: **Compared with the most common diplotype (haplotype pair), the odds ratios for advanced adenoma were 0.80 [95% confidence interval (CI), 0.60-1.06], 0.79 (95% CI, 0.55-1.13), and 0.56 (95% CI, 0.36-0.88) for the other three common diplotypes (>5% frequency). Although calcium intake was inversely associated with adenoma risk, *CASR* diplotypes did not modify this association. However, the power to investigate interactions was limited.**

Conclusion: **Variants in the *CASR* intracellular signaling region were significantly associated with the risk of advanced adenoma.** (Cancer Epidemiol Biomarkers Prev 2004;13(12):2181–6)

Introduction

Several recent cohort studies (1-6) and three randomized intervention trials (7, 8) report inverse associations between calcium intake and colorectal tumors; the calcium-sensing receptor (CASR) may mediate this chemopreventive effect of calcium (9-12). CASR detects extracellular calcium and has a key role in calcium homeostasis and other diverse and cell type–specific functions, including regulation of cell differentiation, proliferation, and apoptosis (9-16). The expression patterns of *CASR* support its involvement in colorectal tumor development; *CASR* expression is high in normal colonic epithelial cells, is lower in well-differentiated colon cancer tissue, and is greatly reduced in undifferentiated carcinomas (9, 17, 18). Consistent with this finding, calcium inhibits the proliferation of well-differentiated but not poorly differentiated cells (19).

CASR has three parts, a large extracellular domain (about 600 amino acids) containing the calcium-binding site, a transmembrane domain (about 250 amino acids) spanning seven subunits, and a carboxyl-terminal intracellular tail (about 220 amino acids) containing the signal transduction region. The intracellular tail of CASR affects several important functions of this receptor, including its level of cell surface expression, the rate at which the CASR desensitizes after calcium exposure, and the capacity of signal transduction into the cell (20, 21). CASR is directly regulated by protein kinase C phosphorylation (22-24), with three of five putative protein kinase C phosphorylation sites located in the intracellular tail (25-27).

Rare mutations, located almost exclusively in the extracellular and transmembrane domain, lead to familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia (28-31). In the search for these rare mutations, common genetic variants (polymorphisms) in *CASR* have been identified (32), which may alter function or expression of *CASR*. We investigated the three common nonsynonymous single nucleotide polymorphisms (SNP) in the coding region of the intracellular *CASR* tail (32) in relation to risk of colorectal adenoma, because of the importance of this region for cellular signal transduction.

We also investigated whether the *CASR* variants modify the effect of calcium on colorectal adenoma. Because the promoter region of *CASR* contains a vitamin D

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receptor (VDR) response element, enabling vitamin D through VDR to regulate *CASR* transcription (33), we also considered interaction between *CASR* polymorphisms, serum vitamin D, and a variant in the *VDR* gene.

Materials and Methods

This case-control study was nested within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO Trial), a multisite investigation (Birmingham, AL, Denver, CO, Detroit, MI, Honolulu, HI, Marshfield, WI, Minneapolis, MN, Pittsburgh, PA, Salt Lake City, UT, St. Louis, MO, and Washington, DC) of the effectiveness of cancer screening, early markers of cancer, and the etiology of cancer (34, 35). Participants, ages 55 to 74 years, who were randomly assigned to the screening group, were offered a flexible sigmoidoscopy examination of the distal colon (60 cm) at study entry. If polyps or other suspect lesions were identified, participants were referred for further colonoscopy and surgery, if indicated. All available medical and pathologic reports on follow-up were obtained and coded by trained medical record abstractors without a centralized pathology review. The institutional review boards of the U.S. National Cancer Institute and the 10 screening centers approved the study and all participants provided informed consent.

Study Population. Cases and controls for this study were drawn from the participants randomly assigned to the screening arm of the PLCO Trial between September 1993 and September 1999, who filled out the risk factor questionnaire, had a successful sigmoidoscopy (insertion to at least 50 cm with >90% of mucosa visible or a suspect lesion identified), and provided a blood sample for use in etiologic studies (n = 42,037). Of these participants, we excluded 4,834 with a self-reported history of ulcerative colitis, Crohn's disease, familial polyposis, colorectal polyps, Gardner's syndrome, or cancer, except basal-cell skin cancer. We randomly selected 772 of 1,234 cases with at least one distal advanced colorectal adenoma ($\geq$1 cm, high-grade dysplasia, or villous elements including tubulovillous adenoma) and 777 of 26,651 control participants, with a negative sigmoidoscopy screening (i.e., no polyp or other suspect lesion), matched to the cases by gender and ethnicity.

Genotype Analysis. DNA was extracted from buffy coat or whole blood samples. We used Taqman (Applied Biosystems, Foster City, CA) to analyze three SNPs in exon 7 of the *CASR* gene, A986S (rs1801725), R990G (rs1042636), and Q1011E (rs1801726). For validation purposes, we resequenced approximately 600 bp upstream and downstream of the three SNPs in a multiethnic group (n = 102; ref. 36). This sequence information was used to design primers for the Taqman assay (36). We only used the Taqman assay if genotype results were 100% concordant with sequencing results for the same 102 individuals (36). Because of insufficient DNA yields, DNA that did not amplify, or failure of unambiguous genotyping for 56 cases and 48 controls, genotype results were available for 716 cases and 729 controls (success rate of 95.1%, 98.3%, and 98.4% for A986S, R990G, and Q1011E, respectively). Genotype results of blinded quality control samples (40 participants assayed two to four times, total 136 results) were 100% concordant. Genotype analysis for the *VDR Taq*I polymorphism was reported in detail elsewhere (37).

Other Factors. At baseline, participants filled out a risk factor questionnaire, including questions on demographic factors, personal, and family medical history, physical activity, weight, height, and smoking. Dietary intake for the year before enrollment was assessed, with a 137-item food frequency questionnaire, including an additional 14 questions on supplement use. To calculate daily nutrient intake from the diet, we multiplied the daily frequency of each food item by the nutrient value of the gender-specific portion size (38) using the U.S. Department of Agriculture nutrient database (39). To calculate total nutrient intake, we combined dietary and supplemental nutrient intake. Details about analysis of serum vitamin D levels (25-hydroxyvitamin D and 1,25 dihydroxyvitamin D) have been reported elsewhere (37).

Statistical Analysis. We tested whether each SNP was in Hardy-Weinberg equilibrium among the controls. As estimates of linkage disequilibrium between SNPs, we calculated D' (40) and r^2 (41). We used PHASE 2.0.2 to reconstruct the most likely pair of haplotypes (diplotype) and their probability for each participant (42, 43). As a global test for association in the genomic region, we used the likelihood ratio statistic to test if the overall frequencies of the most likely diplotypes differed between cases and controls.

Genotype-specific and diplotype-specific prevalence odds ratio (OR) for colorectal adenoma and 95% confidence intervals (CI) were calculated by logistic regression using the genotype or the most likely diplotype as covariate and adjusting for age, screening center, gender, and ethnicity. Other potential risk factors for colorectal tumors (educational attainment, smoking, alcohol intake, aspirin use, ibuprofen use, physical activity, body mass index, energy intake, fiber intake, red meat intake, and folate intake), either separately or together, did not change the risk estimates for *CASR* by >10% and were not included in the analysis.

We investigated whether the associations of colorectal adenoma with calcium intake, vitamin D serum levels, and a *VDR* polymorphism were modified by *CASR* variants, by stratified analyses and tests for multiplicative interaction, with inclusion of cross-product terms in the regression models. The statistical significance of interaction terms was investigated by comparing the log-likelihood statistics of the main effect model with the joint effects model. On the basis of our earlier analysis (2), we adjusted the analysis of association between calcium intake and adenoma risk for age, screening center, gender, ethnicity, education, smoking, alcohol intake, aspirin use, ibuprofen use, physical activity, body mass index, and intake of energy, fiber, red meat, and folate. We adjusted the analysis of association between serum vitamin D levels and adenoma risk for age, screening center, gender, ethnicity, month of blood draw (37).

Results

Advanced adenoma cases were slightly older and less well-educated than controls (Table 1), the distributions

Table 1. Characteristics of the study population

Characteristics	Cases	Controls
N	716	729
Age, *n* (%)		
55-59 years	237 (33.1%)	336 (46.1%)
60-64 years	230 (32.1%)	186 (25.5%)
65-69 years	155 (21.7%)	136 (18.7%)
70-74 years	94 (13.1%)	71 (9.7%)
Female, *n* (%)	215 (30.3%)	228 (31.3%)
Ethnic origin, *n*		
Non-Hispanic Black	21 (2.9%)	23 (3.2%)
Non-Hispanic White	673 (94.0%)	684 (93.8%)
Others	22 (3.1%)	22 (3.0%)
Education, *n* (%)		
<12 years of school	68 (9.5%)	47 (6.5%)
12 years of school/high school equivalent	170 (23.7%)	170 (23.3%)
Some college	258 (36.0%)	235 (32.2%)
College and above	220 (30.7%)	277 (38.0%)

by gender and ethnic origin were similar because we matched on these variables. About 94% of the study participants were non-Hispanic White and 69% were male.

The minor allelic frequency in controls was 14.5%, 8.5%, and 5.6% for A986, R990, and Q1011, respectively. All three genotypes were in Hardy-Weinberg equilibrium ($P = 0.92$ for A986S, $P = 0.69$ for R990G, and $P = 0.62$ for Q1011E, calculated for non-Hispanic White controls). No single genotype was significantly associated with risk for advanced colorectal adenoma (Table 2).

Four haplotypes explained 100% of the genetic variation: haplotype 000 consisted of the three common variants (A986, R990, Q1011); haplotypes 100, 010, and 001 were characterized, respectively, by one polymorphic variant at codon 986, 990, and 1011 (Table 3). Each phase call was assigned with more than 95% probability. The probability of the most likely haplotype pair (diplotype) was >0.99 for 93.8% of the participants, >0.95 for 95.1% of the participants, and >0.80 for 99.3% of the participants. Four common diplotypes (000/000, 000/100, 000/010, and 000/001) covered 91.4% of the study population. Because the frequency of any of the other six possible diplotypes was low (<2.5%), we did not assess adenoma risk associated with these diplotypes separately. Overall, the frequency of the four common diplotypes differed between cases of advanced colorectal adenoma and controls (likelihood ratio test 9.012, 4 *df*, $P = 0.06$). Compared with participants carrying the most common diplotype (000/000), adenoma risk was significantly reduced for participants carrying the 000/001 diplotype (OR 0.56) and tended (nonsignificantly) to be reduced for carriers of the 000/100 and 000/010 diplotypes (Table 4). This analysis is equivalent to comparing heterozygotes for a given polymorphism with a reference group that is a homozygote for the common allele of all three variants. Excluding participants with a probability for the most likely haplotype of <0.99 ($n = 90$) resulted in similar risk estimates as shown in Table 4: (000/100) versus (000/000) OR 0.77, 95% CI, 0.58-1.02; (000/010) versus (000/000) OR 0.78, 95% CI, 0.54-1.12; and (000/001) versus (000/000) OR 0.51, 95% CI, 0.33-0.78.

We found a protective association between total calcium intake and advanced colorectal adenoma risk, with a 21% risk reduction per 1,000 mg increase in calcium intake (OR 0.79, Table 5). This association was similar to results for the entire study population, which also showed that a linear coefficient fits well with the calcium-adenoma association (2). Stratification by *CASR* diplotype resulted in similar protective calcium-adenoma associations for participants carrying 000/000 or 000/100. The association between calcium intake and advanced adenoma risk varied for participants carrying 000/010 or 000/001 diplotypes, but the small number of cases and controls resulted in less precise risk estimates. No multiplicative interaction coefficients between total calcium intake and the *CASR* diplotypes were below $P = 0.2$. Furthermore, we found that the association between serum vitamin D levels or *VDR TaqI* genotype and adenoma risk did not substantially vary by *CASR* diplotype (data not

Table 2. Association between genotypes of *CASR* and advanced colorectal adenomas

CASR genotype	Cases, *n* (%)	Controls, *n* (%)	OR (95% CI)
A986S			
GG	545 (76.8)	531 (73.1)	1.00
GT	148 (20.9)	179 (24.7)	0.82 (0.64-1.06)
TT	17 (2.4)	16 (2.2)	1.07 (0.52-2.19)
R990G			
AA	590 (85.1)	580 (83.9)	1.00
AG	94 (13.6)	104 (15.1)	0.92 (0.68-1.26)
GG	9 (1.3)	7 (1.0)	1.17 (0.36-3.79)
Q1011E			
CC	646 (91.1)	647 (89.1)	1.00
CG	58 (8.2)	77 (10.6)	0.76 (0.53-1.11)
GG	5 (0.7)	2 (0.3)	2.35 (0.44-12.6)

NOTE: ORs adjusted for age, gender, ethnic origin, and study center.

Table 3. Haplotype frequency of *CASR* reconstructed from genotype data

Haplotype	Cases, frequency (SE)	Controls, frequency (SE)
000 = A986, R990, Q1011	0.743 (0.0025)	0.741 (0.0026)
100 = 986S, R990, Q1011	0.128 (0.0010)	0.145 (0.0011)
010 = A986, 990G, Q1011	0.081 (0.0021)	0.085 (0.0024)
001 = A986, R990, 1011E	0.048 (0.0012)	0.056 (0.0007)

NOTE: A986S and R990G, $r^2 = 0.018$, $D' = -0.99$, $P = 0.002$; A986S and Q1011E, $r^2 = 0.010$, $D' = -1.00$, $P = 0.0003$; R990G and Q1011E, Q1011E, $r^2 = 0.004$, $D' = -0.86$, $P = 0.13$.

Table 4. Association between diplotypes of *CASR* and advanced colorectal adenomas

CASR diplotype	Cases (*n*)	Controls (*n*)	OR (95% CI)
000/000	410	369	1.00
000/100	132	151	0.80 (0.60-1.06)
000/010	71	84	0.79 (0.55-1.13)
000/001	39	64	0.56 (0.36-0.88)
All other diplotypes*	63	61	0.99 (0.67-1.46)

NOTE: ORs adjusted for age, gender, ethnic origin, and study center.
000 = A986, R990, Q1011; 100 = 986S, R990, Q1011; 010 = A986, 990G, Q1011; 001 = A986, R990, 1011E.
*Frequency of any of the other six possible diplotypes was <2.5%

shown, main effects of vitamin D and *VDR* were reported elsewhere; ref. 37).

Risk estimates presented here included all participants and were adjusted for ethnic origin. Restricting the analysis to non-Hispanic Whites resulted in risk estimates very similar to those presented (data not shown).

Discussion

We found a significant association between *CASR* diplotypes and advanced colorectal adenoma. Risks were not specific to one of the three genotypes studied, suggesting that the association between genetic variants in *CASR* and adenoma risk may depend on the diplotype patterns studied, or linked genetic variants upstream or downstream of these three SNPs.

CASR is suggested to be important for mediating the anticarcinogenic effect of calcium (9-12). Lamprecht and Lipkin (12) recently reviewed the molecular mechanisms of CASR in colorectal cancer, indicating that specific signaling pathways involved in cell growth and differentiation are activated by calcium through CASR, including promotion of E-cadherin expression, suppression of β-catenin/T cell factor (9) activation, and activation of the p38 mitogen-activated protein kinase cascade (44). The intracellular signaling region of CASR which we studied interacts with filamin-A, linking CASR-mediated activation to the mitogen-activated protein kinase cascades (45, 46). CASR is expressed on both the basolateral and luminal surfaces, indicating that CASR senses changes in calcium concentrations in the colon lumen as well as in blood (12, 47, 48); and Kalley et al. (10, 11) showed that the cell proliferative effect of low intestinal calcium concentrations is likely mediated by CASR via protein kinase C–signaling activation leading to up-regulation of c-myc expression. Low calcium levels can occur particularly in the large intestine due to increased fractional absorption of calcium in the small intestine—from 20% to 70%—if calcium intake is low.

From an evolutionary standpoint, the R990 (common) variant seems functionally relevant, as evidenced by cross-species evolutionary conservation (49). Based on physical principles, the change from arginine (R) to glycine (G) at codon 990 results in a radical amino acid change, from a positively charged to a hydrophilic base, whereas the amino acid changes in the two other SNPs are more conservative ($Ala^{986}Ser$, hydrophobic to hydrophilic; $Gln^{1011}Glu$, hydrophilic to negatively charged; ref. 50). Thus, 990G could be related to altered function in CASR in a manner that influences risk for colorectal adenoma, however, our study does not point specifically to the R990G polymorphism, rather it suggests that one or more of the minor variants (986S, 990G, 1011E), or other unmeasured polymorphisms in linkage disequilibrium with them, are associated with reduced risk for colorectal adenoma.

Table 5. Association between calcium (1,000 mg/day) and advanced colorectal adenoma stratified by *CASR* diplotype

CASR diplotype	Calcium intake, OR (95% CI)
Overall	0.79 (0.61-1.02)
000/000	0.68 (0.47-0.99)
000/100	0.69 (0.39-1.23)
000/010	1.49 (0.57-3.90)
000/001	0.36 (0.07-1.88)
Other diplotypes	0.97 (0.28-3.36)

NOTE: ORs adjusted for age, gender, ethnic origin, study center, energy intake, educational attainment, tobacco use, alcohol intake, aspirin and ibuprofen use, physical activity, body mass index, and intake of red meat, folate, and dietary fiber. Range of the 10th to 90th percentile of calcium intake in controls (567-2,021 mg/day).

Ours is the first study to evaluate *CASR* variants in relation to colorectal adenoma risk. A small study investigated the association between *CASR* A986S and rectal cancer in 56 cases and 112 controls, however, the data in two reports (51, 52) are conflicting, and it is not clear whether the A986S variant influences the risk for rectal cancer.

There is growing experimental (53) and epidemiological evidence (1-8), including recent evidence from this study population, that high calcium intake protects from colorectal tumors (2). This study shows no substantial interrelation between *CASR* gene variants, calcium intake and colorectal adenoma risk. Our study is limited by sample size for the evaluation of *CASR*-calcium interactions; however, the negative results might indicate that the gene variants in the intracellular signaling region operate functionally independently from the extracellular part of this receptor, which binds calcium. Some studies showed lower serum calcium levels in homozygous A986 carriers (55-57), whereas others did not find an effect on serum calcium concentrations (58-60).

It is widely recognized that vitamin D induces differentiation and apoptosis in normal and tumor colonic cells (12), and a small number of observational studies including ours (37) suggest a reduced risk of colorectal tumors with increasing levels of serum vitamin D. We found no substantial interaction for vitamin D and *VDR* genotype with *CASR* genotypes or diplotypes; however, such interactions may be more likely with genetic variants in the promoter region of *CASR*, where the VDR response element is located.

We focused on our analysis of left-sided adenoma. Because differences in the associations with right-sided and left-sided colorectal neoplasia have been reported for some risk factors, including calcium (5, 8, 61), the extrapolation of our findings with all adenomas (right-sided and left-sided) or right-sided adenoma should be done with caution.

Ninety of the cases in our analysis had a hyperplastic polyp as well as an advanced adenoma. Because the association between *CASR* diplotypes and advanced adenoma with and without hyperplastic polyps was similar (data not shown), the observed *CASR* diplotype-adenoma association cannot be explained by the inclusion of adenoma cases with concomitant hyperplastic polyps.

We used the most likely pair of haplotypes in the standard logistic regression analysis to estimate relative risk. Because of the inaccuracy in haplotype imputation (62), our approach could potentially underestimate the true effect. Such an error could bias the risk estimate if the true effect of the gene was due to a haplotype and not to specific variants. However, given the rarity of the alleles

and the strong linkage disequilibrium, there was little ambiguity in imputing haplotype pairs for each participant and the possibility of such error in our analysis was minimal. Furthermore, excluding participants with <0.99 probability of the most likely haplotype pair did not change the risk estimates for *CASR* diplotypes.

The study design enabled us to randomly select cases and controls derived from the same source population and screened following a standardized procedure, in particular, cases were not screened based on symptoms. The large number of cases identified in the study population allowed us to focus our analysis on advanced adenoma. Because these lesions are more likely to progress to colorectal cancer, they are a particularly meaningful intermediate outcome for studying risk factors for colorectal cancer. Cases were identified from the initial baseline screening, so we cannot distinguish the effect of genetic variants in *CASR* on the temporal component of adenoma formation.

In conclusion, we found a significant association between advanced colorectal adenoma and *CASR* diplotypes comprised of three nonsynonymous polymorphic variants in the intracellular signaling region of *CASR*, supporting laboratory evidence suggesting a major role for CASR in the chemopreventive effects of calcium. It will be important to further investigate genetic variations in *CASR* in separate study populations, expanding genetic characterization to the entire functional region of *CASR*, including the calcium binding and the vitamin D–related regulatory regions, and to more fully characterize the gene variant-function correlates of this gene.

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